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CELL, vol. 47, no. 2, pages 207-216; M.D. BEEN et al.: "One binding site determines sequence specificity of tetrahymena pre-rRNA self-splicing, trans-splicing, and RNA enzyme activity"

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Science, Volume 224, Issued 1984 May, (Washington, D.C., U.S.A.), (A. ZAUG et al), a Labile Phosphodiester Bond at the Ligation Junction in a Circular Intervening Sequence RNA", see pages 574-578.

Biochemistry, Volume 25, Issued 1986, August, (Washington, D.C., U.S.A.), (B. BASS et al) "Ribozyme Inhibitors: Deoxyguanosine and Dideoxyguanosine are Competitive Inhibitors of Self Splicing of the Tetrahymena Ribosomal Ribonucleic Acid Precursor". See pages 4473-4477.

Proceedings National Academy of Sciences, Volume 83, Issued 1986, June (Washington, D.C., U.S.A.), (T. CECH), "a Model for RNA-catalyzed Replication of RNA". See pages 4360-4363.

Description

This invention concerns compositions of RNA functioning as an RNA enzyme, i.e. a ribozyme with sequence-specific endoribonuclease activities.

5 The *Tetrahymena* rRNA intervening sequence (IVS) is a catalytic RNA molecule or ribozyme. It mediates RNA self-splicing, accomplishing its own excision from the large ribosomal RNA precursor, and subsequently converts itself to a circular form (Kruger, K., *et al* (1982) *Cell* 31:147-157; Zaug, A.J., *et al* (1983) *Nature* 301:578-583). In these reactions, the splice sites and cyclization sites can be viewed as intramolecular substrates for an activity that resides within the IVS RNA (Zaug, A.J., *et al* (1984) *Science* 224: 574-578). This is not a true enzymatic reaction however since the RNA is not regenerated in its original form at the end of the self-splicing reaction. The IVS RNA when in its linear form is referred to as L IVS RNA.

This view has been validated by studies of the L-19 IVS RNA, a linear form of the IVS which is missing the first 19 nucleotides. Because it lacks the cyclization sites, the L-19 IVS RNA is inhibited from undergoing intramolecular reactions (Zaug, A.J., *et al* (1984) *Science* 224: 574-578). It still retains activity, however, and can catalyze cleavage-ligation reactions on other RNA molecules (Zaug, A.J. and Cech, T.R. (1986) *Science* 231:470-475). When provided with oligo(cytidylic acid) as a substrate, the L-19 IVS RNA acts as an enzyme with nucleotidyltransferase [poly(C) polymerase] and phosphodiesterase (ribonuclease) activities (Zaug, A.J. and Cech, T.R. (1986) *Science* 231: 470-475). With 3'-phosphorylated oligo(C) substrates, the same ribozyme acts as a phosphotransferase and an acid phosphatase (Zaug, A.J. and Cech, T.R. (1986) *Biochemistry* 25:4478-4482). A key mechanistic feature of all four of these reactions is the formation of a covalent enzyme-substrate intermediate in which a nucleotide or phosphate is esterified through the 3'-O of G414, the 3' terminal guanosine of the IVS RNA. This covalent intermediate is formed by nucleophilic attack by the 3'-hydroxyl group of G414. The self-splicing mechanism is further reviewed by Cech (1986) *Scientific American* 225 (5):64-75.

Szostak (1986) *Nature* 322: 83-88 discloses that 5' and 3' deletions of the *Tetrahymena* IVS are capable of acting on substrate molecules comprising the 5' portion of the *Tetrahymena* IVS.

DESCRIPTION OF THE DRAWINGS

- 30 Figure 1 compares RNA self-splicing (A) to RNA endoribonuclease activity (B).
 Figure 2 shows products of cleavage of a variety of RNA substrates by the RNA endoribonuclease.
 Figure 3 compares different substrate activity of three variant forms of L-19 IVS ribozyme in 2.5M urea.
 35 Figure 4 shows the time course of oligonucleotide cleavage.
 Figure 5 shows the plasmid construction which produces the L-21 IVS RNA.

DETAILED DESCRIPTION OF THE DRAWINGS

40 Figure Legends

Fig. 1 A model for the L-19 IVS RNA acting like an RNA restriction endonuclease by a mechanism that is an intermolecular version of the first step of pre-rRNA self-splicing. Thin letters and lines represent IVS sequences, boldface letters and thick lines represent exon sequences (above) or substrate RNA sequences (below), and the G in italics is a free guanosine nucleotide or nucleoside.

45 Fig. 2 The L-19 IVS-beta RNA cleaves large RNA substrates with transfer of guanosine. a, Uniformly labeled 0.6 μ M (u = micro) pAK105 RNA (508 nt) incubated with 0.2 μ M L-19 IVS-beta RNA and 0, 0.1 or 0.5 mM GTP (unlabeled) for 1 h under conditions described below. (M) Mixture of 4 substrate RNAs as molecular weight markers. b, Various tritiated RNA substrates (1 μ g each) incubated with 0.2 μ M L-19 IVS-beta RNA and 120 μ M [α - 32 P]GTP for 1 h under the same reaction conditions as a. Autoradiogram reveals [32 P]GTP-labeled products only. The L-19 IVS-beta RNA also becomes GTP-labeled during the incubation. c, Nucleotide sequence of the cleavage product pAK105(1) determined by the enzymatic method (Donis-Keller, H., (1980) *Nucleic Acids Res.* 8:3133-3142). Some nucleotides could not be assigned due to presence of a band in the untreated (control) RNA sample. G*, labeled GTP joined to the RNA during the reaction.

55 Methods: L-19 IVS RNA was synthesized by methods similar to those described previously (Zaug, A.J., and Cech, T.R., (1986) *Science* 231:470-475), except a different RNA polymerase-template system was used. Plasmid pT7-TT1A3 (which contains a T7 RNA polymerase promoter, a 42 bp 5' exon, the entire 413

bp IVS, and an 82 bp 3' exon) was cleaved with Eco RI and transcribed with bacteriophage T7 RNA polymerase (Davanloo, P., et al. (1984) *Proc. Nat'l. Acad. Sci. U.S.A.* 81: 2035-2039). The transcripts were incubated further under self-splicing, cyclization, and site-specific hydrolysis conditions to produce L-19 IVS RNA (Zaug, A.J., et al. (1984) *Science* 224:574-578; Zaug, A.J. and Cech, T.R., (1986) *Science* 231:470-475). The 3'-terminal guanosine was then removed by periodate oxidation and beta-elimination (Winter, G., et al. (1978) *Nucleic Acids Res.* 5, 3129-3139) to yield L-19 IVS-beta RNA. Substrate RNAs were produced by T7 RNA polymerase transcription of BamHI-cleaved pAK105 (Mount, S.M., et al. (1983) *Cell* 33:509-518), XmnI- or ScaI-cleaved pT7-1 (purchased from U.S. Biochemical Corp.), and SnaBI-cleaved pDW27, which encodes M1 RNA (obtained from D. Wahl and N. Pace). Substrate RNAs were incubated with L-19 IVS-beta RNA in 5 mM MgCl₂, 10 mM NaCl, 50 mM Tris-HCl, pH 7.5 at 50 °C; in addition, GTP was present at the concentration indicated. Reactions were stopped by the addition of EDTA to a final concentration of 25 mM. Products were analyzed by electrophoresis in 4% polyacrylamide, 8 M urea gels and subjected to fluorography (a) or autoradiography (b).

Fig. 3 Three different ribozymes can distinguish between sequences that differ by only one single-base change within the recognition element. a, Synthesis of defined oligoribonucleotide substrates by the method of Lowary et al. (Lowary, P., et al. *NATO ASI Series A*, vol. 110, 69-76, 1986). DNA was transcribed with T7 RNA polymerase, in the presence of [α -³²P]ATP to produce oligoribonucleotides labeled with ³²P in the positions indicated (*). b, Proposed interactions between the three oligoribonucleotide substrates (top strands; boldface letters) and the active sites of the matched ribozymes (bottom strands). Arrows designate sites of cleavage and guanosine addition. c, Cleavage of 3 oligoribonucleotide substrates by wild-type and variant L-19 IVS RNAs, assayed by 20% polyacrylamide, 7M urea gel electrophoresis: (-): untreated substrate. In other pairs of lanes, 1.0 uM ³²P-labeled substrate was incubated with 0.125 M ribozyme for 15 min (left lane) or 60 min (right lane) at 50 °C in 10 mM MgCl₂, 10 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 mM GTP (unlabeled), 2.5 M urea. Because the ³²P is confined to the nucleotides downstream from the cleavage site, only the smaller of the products is apparent. The identity of the GA₆ product was confirmed by treatment of the substrate and reaction mixtures with RNase T₂ and monitoring the transfer of ³²P to Gp. The band migrating above GA₆ in lanes 2 and 3 was not produced consistently and has not been identified.

Methods: Substrates were prepared by transcription of deoxyoligonucleotides synthesized on an Applied Biosystems DNA Synthesizer. The same promoter top strand was used in each transcription. The bottom strand, which contains promoter and template sequences, was varied to obtain the different RNA substrates. The DNA was transcribed with purified phage T7 RNA polymerase (Davanloo, P., et al. (1984) *Proc. Nat'l. Acad. Sci. U.S.A.* 81:2035-2039) as described (Lowary, P., et al. *NATO ASI Series*, vol. 110, as above). Variant ribozymes are described by Been and Cech (Been, M.D., et al. (1986) *Cell*, 47:207-216). The 24C ribozyme was similarly prepared from transcripts of pBG/3G:24C. The variant ribozymes were not subjected to beta-elimination to remove their 3'-terminal G.

Fig. 4 Kinetic analysis of the RNA endoribonuclease reaction. a, The oligoribonucleotide substrate (2.5 uM) was incubated with wild-type L-19 IVS-beta RNA (0.2 uM) as in Fig. 3; except that urea was omitted. b, Kinetics of cleavage of GGCCUCUA₆ as a function of GTP concentration. RNA substrate concentration was kept constant at 2.5 uM. c, Kinetics of cleavage as a function of RNA substrate concentration, with GTP concentration kept constant at 0.5 mM.

Methods: Products were separated by polyacrylamide gel electrophoresis. With the autoradiogram as a guide, each gel was cut into strips; and the radioactivity in the unreacted substrate and the GA₆ product was determined by liquid scintillation counting. The initial velocity of cleavage (V₀) was determined from a semilogarithmic plot of the fraction of reaction as a function of time. 1/V₀ was then plotted as a function of inverse substrate concentration; the graphs are linear least-squares fits to the data points.

Fig. 5 Plasmid pBGST7 contains a fragment with the IVS inserted into the multicloning site of a small pUC18 derivative which in turn contains a phage T7 promoter. The double line represents the plasmid DNA with the IVS. The relative positions of the promoters are indicated. The positions of the EcoRI and Hind III site are indicated by the arrowheads. The upper line represents the in vitro transcript made from purified plasmid DNA with phage T7 RNA polymerase. The numbers above it refer to the length, in nucleotides, of the exons and IVS. The lower line represents the in vivo transcript of the 5' end of lacZ'. The IVS (heavy line) contains stop codons in all three reading frames, so a functional alpha fragment can only be produced if the IVS is excised and the exons are joined by splicing in *E. coli*.

We describe here a fifth enzymatic activity of the *Tetrahymena* ribozyme. It cleaves other RNA molecules at sequences that resemble the 5' splice site of the rRNA precursor. Cleavage is concomitant with addition of a free guanosine nucleotide to the 5' end of the downstream RNA fragment; thus, one product can be readily end-labeled during the reaction. The reaction is analogous to the first step of pre-rRNA self-splicing (Fig. 1). Cleavage does not require nucleotide G⁴⁴ of the ribozyme; thus, unlike the first

four activities, it does not involve formation of a covalent enzyme-substrate intermediate. Thus there exists as a result of the work of the invention sequence-specific endoribonucleases; protein-free i.e. able to act in the absence of protein, and composed of RNA, which are enzymatically active on other RNA molecules. These RNA ribozymes act on exogenous RNA. Thus the enzyme or ribozyme is composed of RNA and the substrate is RNA (or mixed RNA-DNA polymers).

The ribozyme has high specificity for cleavage after the nucleotide sequence CUCU; under stringent conditions it can discriminate against sites that have a 3-out-of-4 match to this recognition sequence. For example, in a solution containing 2.5 M urea the ribozyme cleaves after CUCU while ignoring the related sequences CUGU and CGCU (Fig. 3c). The sequence specificity approaches that of the DNA restriction endonucleases (Nathans, D. and Smith, H.O. (1975) *Annu. Rev. Biochem.* 44:273-293). We further show that site-specific mutations in the active site of the IVS RNA, the so-called internal guide sequence (Davies, R.W., et al. (1982) *Nature* 300:719-724; Waring, R.B., et al. (1986) *Nature* 321:133-139) or 5' exon-binding site (Inoue, T., et al. (1985) *Cell* 43:431-437; Garriga, G., et al. (1986) *Nature* 322:86-89; Bech, M.D. and Cech, T.R., (1988) *Cell* 47, 207-216), alter the sequence specificity of the ribozyme in a predictable manner. In its endoribonuclease mode, the L-19 IVS RNA recognizes four or more nucleotides in choosing a reaction site. Protein ribonucleases that are active on single-stranded RNA substrates have specificity only at the mononucleotide level (for example, ribonuclease T₁ cleaves after guanosine). Thus the L-19 has more base-sequence specificity for single-stranded RNA than any known protein ribonuclease, and may approach the specificity of some of the DNA restriction endonucleases. An attractive feature of this new RNA endoribonuclease is that its substrate specificity can be completely and predictably changed by altering the sequence of the internal binding site.

The endoribonuclease reaction is analogous to the first step of pre-rRNA self-splicing (Fig. 1). Both the enzymatic and the self-splicing reactions make use of the same two binding sites, an oligopyrimidine-binding site and a guanosine-binding site, to achieve specificity and to contribute to catalysis. The oligopyrimidine-binding site is also known as the 5' guide sequence (Waring, R.B., et al. (1986) *Nature* 321:133-139) or the 5' exon-binding site (Inoue, T., et al. (1985) *Cell* 43:431; Garriga, G., et al. (1986) *Nature* 322:86-89; Bech, M.D. and Cech, T.R., *Cell* (1986) 47, 207-216). Its role in self-splicing has been conclusively demonstrated by the analysis of single-base mutations and second-site suppressor mutations (Waring, R.B., et al. (1986) *Supra*; Bech, M.D. and Cech, T.R., (1988) *Supra*; Perea, J. and Jacq, C., (1985) *EMBO, J.* 4:3281). The role of these same nucleotides in the endoribonuclease reaction is demonstrated by the change in substrate specificity of the mutant enzymes (Fig. 3); the altered specificity being predictable by the rules of Watson-Crick base pairing. The guanosine-binding site involved in self-splicing has not been localized to a particular set of nucleotides, but its general features have been described (Bass, B.L. and Cech, T.R., (1984) *Nature* 308:820; (1986) *Biochemistry* 25:4473). The endoribonuclease activity appears to make use of the same guanosine-binding site by the following criteria: in both cases guanosine is as active as GTP, whereas UTP, CTP, ATP and dGTP have little if any activity. In addition, the K_m of 44 μ M for GTP shown is in reasonable agreement to the value of 32 ± 8 μ M determined for self-splicing under somewhat different reaction conditions (Bass, B.L. and Cech, T.R., *Biochemistry* (1986) *Supra*).

The endoribonuclease activity of the L-19 IVS RNA does not require its 3'-terminal guanosine (G^{+14}). In this respect it differs from the nucleotidyl transfer, phospho transfer and hydrolytic activities of the same enzyme. In those reactions G^{+14} participates in a covalent enzyme-substrate complex that appears to be an obligatory reaction intermediate (Zaug, A.J. and Cech, T.R. (1986) *Science* 231:470-475; Zaug, A.J. and Cech, T.R. (1986) *Biochemistry* 25:4478). Thus, the L-19 IVS RNA is not restricted to reaction mechanisms involving formation of a covalent enzyme-substrate intermediate. It can also catalyze bisubstrate reactions by a single-displacement mechanism. Ribonuclease P, an RNA enzyme that catalyzes cleavage by hydrolysis rather than by transesterification, also appears to act without formation of a covalent intermediate (Marsh, T.L., et al. (1985) *Science* 229:79-81; Guerrier-Takeda, C., et al. (1988) *Biochemistry* 25:1509).

The L-19 IVS RNA endoribonuclease activity reported here appears to require single-stranded RNA substrates. Based on work recently reported by Szostak ((1986) *Nature* 322:83-88), it seems possible that a smaller version of the *Tetrahymena* IVS RNA missing its 5' exon-binding site may have an endoribonuclease activity that requires a base-paired substrate. The substrate tested by Szostak ((1986) *Nature* *Supra*) was an RNA fragment containing the end of the 5' exon paired with the 5' exon-binding site. However, this RNA "substrate" also included a substantial portion of the IVS RNA, so it remains to be established whether the ribozyme has endoribonuclease activity with double-stranded RNA substrates in general.

The present invention provides an enzymatic ribonucleic acid molecule which is capable of cleaving by transesterification a separate RNA molecule at a predetermined phosphate ester bond in a single-stranded target nucleotide sequence within the separate RNA molecule, which enzymatic molecule comprises:

- (i) a substrate binding portion capable of binding with the target nucleotide sequence; and
- (ii) an enzymatic portion having endonuclease activity independent of any protein *in vitro*, wherein said enzymatic molecule can only cleave the single-stranded target sequence without forming a covalent bond between said enzymatic molecule and any portion of said separate RNA molecule.

5 The invention further provides a method for specifically cleaving a separate RNA molecule at a target nucleotide sequence comprising single-stranded RNA, comprising contacting an enzymatic ribonucleic acid molecule as defined above with a separate RNA molecule which contains a single-stranded target nucleotide sequence under conditions to cause specific cleavage of the target sequence by endonuclease activity of said enzymatic molecule.

10 The invention also provides a method for producing an enzymatic ribonucleic acid molecule having an endonuclease activity independent of any protein, said activity being specific for a nucleotide sequence defining a cleavage site comprising a single-stranded RNA target site in a separate first RNA molecule, comprising the steps of:

- (i) identifying a second RNA molecule capable of intramolecular cleavage by transesterification resulting from nucleophilic attack at a phosphorous ester bond,
- (ii) determining the cleavage site and nucleophilic attacking group of said second RNA molecule, said site comprising at least four bases, and
- (iii) synthesising an enzymatic ribonucleic acid molecule corresponding to said second RNA molecule but lacking said cleavage site and said nucleophilic attacking group, such that said enzymatic molecule can only cleave by transesterification, a single-stranded target sequence without forming a covalent bond between itself and any portion of the target sequence, wherein said synthesising optionally includes altering one or more residues of said second RNA molecule such that the target specificity of the enzymatic molecule is altered.

25 Sequence-specific endoribonucleases might have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans, D. and Smith, H.O., (1975) Ann. Rev. Biochem. 44:273). For example, the pattern of restriction fragments could be used to establish sequence relationships between two related RNAs, and large RNAs could be specifically cleaved to fragments of a size more useful for study. The 4-nucleotide specificity of the ribozyme is ideal for cleavage of RNAs of unknown sequence; an RNA of random sequence would have an average of 1 cleavage site every 256 bases. In addition, the automatic end-labelling of one fragment during ribozyme cleavage is a practical advantage.

35 Development of the ribozymes as useful tools for molecular biology has begun. The efficiency of cleavage of large RNA substrates needs to be increased so that complete digests rather than partial digests can be obtained. The effects of denaturants such as urea and formamide must be further explored; they appear to increase the sequence specificity of cleavage, and at the same time they should melt structure in the substrate to maximize the availability of target sequences. Finally, mutagenesis of the active site of the ribozyme by those skilled in the art can be accomplished to ascertain all possible permutations of the 256 possible tetranucleotide cleavage enzymes.

40 RNA sequence recognition. Protein ribonucleases can cleave RNA substrates with high specificity by recognizing a combination of RNA structure and sequence, with the emphasis on structure (e.g., RNase III (Robertson, H.D. (1982) Cell 30:669) and RNase M5 (Stahl, D.A., et al. (1980) Proc. Natl. Acad. Sci. USA 77:5644). Known proteins that cleave single-stranded RNA substrates, on the other hand, have specificity only at the mononucleotide or dinucleotide level (e.g., RNase T₁ cleaves after guanosines [Egami, F., et al. (1980) Molec. Biol. Biochem. Biophys. 32:250-277]. Thus, the L-19 IVS RNA has considerably more base-sequence specificity for cleaving single-stranded RNA than any known protein ribonuclease.

Variant Ribozymes (or other versions of the ribozyme that retain activity)

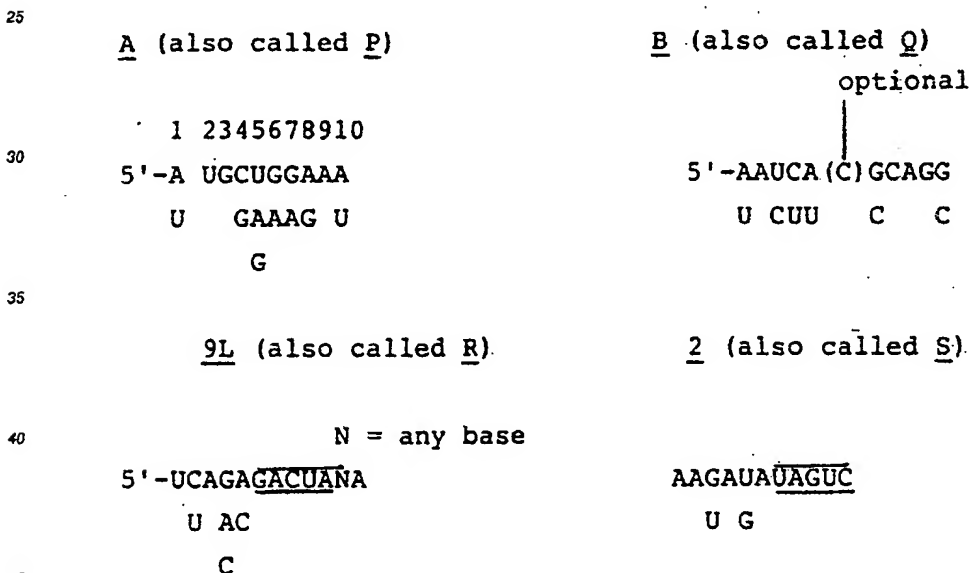
50 Earlier work on sequence requirements for self-splicing (Price, J.V., et al. (1985) Nucleic Acid. Res. 13:1871) show that sequence requirements can be examined as shown therein by insertions and deletions to obtain other self-splicing IVS RNA's. In like manner, we could alter the L-19 IVS RNA to obtain an array of RNA sequence-specific endoribonuclease molecules. Thus three regions were found by Price et al. to be necessary for IVS self-splicing. Similar experiments would reveal necessary portions of L-19 IVS RNA for endoribonuclease activity. Burke, J.M., et al. (1986) Cell 45:167-176 show the role of conserved elements for the IVS self-splicing sequence; that work shows a further use of mutagenesis experiments to alter the highly conserved sequences to alter activity thereof. Just so, in like manner, the activity of the L-19 IVS RNA can be altered with accompanying alteration in activity to effect an array of endoribonucleases.

Cech, T.R., et al. have recently found a yet smaller piece of the L-19 IVS RNA which contains full enzymatic activity and comprises nucleotides 19-331 of the RNA. It is also found that the 21-331 piece is fully active. Plasmids have been constructed to produce the L-19 and L-21 IVS RNA strands directly. Here the promoter is moved to the 19 position or 21 position and the DNA coding for the restriction site is at the 331 position instead of the 414 site.

Been, M.D. and Cech, T.R. ((1986) Cell 47:207) show alteration in the specificity of the polymerase activity to effect polymerase activity with respect to oligo U using site-specific mutagenesis. Thus those skilled in the art can readily use the above to obtain other active L-19 IVS RNA enzymes.

Waring, R.B. and Davies, (1984) Gene 28:277 show a class of IVS RNA molecules with similar structure. This work is similar to that of Cech, T.R., et al. (1983) Proc. Natl. Acad. Sci. USA 80:3903 showing a class of fungal mitochondrial RNA IVS molecules. Some of these other IVS molecules have been found to be self-splicing. (Cech, T.R., et al. (1981) Cell 27:487; Kruger, K., et al. (1982) *ibid*: 31:147; Garriga, G., et al. (1984) *ibid* 39:631; Van der Horst, G., et al. (1985) *ibid* 40:759; Chin, F.K., et al. (1985) J. Biol. Chem. 260:10680; Peebles, C.L., et al. Cell in press; Van der Veen, R., et al., *ibid*. in press) Thus a series, or many series or class, or family of endoribonucleases from the same or other natural sources can be based on the work of the invention. Those skilled in the art will be able to search out other RNA enzymes from various natural sources.

The following RNA sequence elements can be considered to provide the minimum active site for ribozyme activity, based on (Cech, et al., PNAS (1983) and Waring & Davies *Supra*). Elements A and B interact with each other, as do 9L and 2. In many positions of the sequence, more than 1 base is allowed; the observed substitutions are shown by printing a letter directly below the base for which it can substitute. For example, at position 1 in sequence element A, the nucleotides A and U are observed; A being more common.



these pair namely GACUA on left and UAGUC on right as underlined; compensatory base changes are allowed - see Burke, et al. (1986) *Supra*

The linear sequence of non-template DNA coding for L IVS RNA is shown below. Coding for the L-19 IVS RNA "ribozyme" begins at the site indicated by the arrow and extends to the end (G⁺). Of course, in RNA the T's are U's.

L - 19

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5  GAAATAGCAATATTTACCTTTGGAGGGAAGTTATCAGGCATGCACCTGGTA  53
    22 23 24 25
10 GCTAGTCTTTAAACCAATAGATTGCATCGGTTAAAAGGCAAGACCGTCAAA  105
    22 23 24
15 TTGCGGGAAAGGGGTCAACAGCCGTTCACTACCAAGTCTCAGGGGAACTTT  157
    22 23 24
20 GAGATGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACC  209
    22 23 24
25 ACGCAGCCAAGTCTTAAGTCAACAGATCTTCTGTTGATATGGATGCAGTTCA  261
    22 23 24
30 CAGACTAAATGTCGGTCGGGGAAGATGTATTCTTCTCATAAGATATAGT  310
    22 23 24
35 CGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTATGCAACACTGGAGCC  362
    22 23 24
40 GCTGGAACATAATTTGTATGCGAAAGTATATTGATTAGTTTGGAGTACTCG  414
    22 23 24

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In the discussion of L-19 IVS RNA, the region of the active site sequence discussed with respect to activity and variants is:

U U G G A G G G
20 21 22 23 24 25 26 27

The first ribonucleotide for the L-19 IVS RNA at the 5'OH end is the equivalent of the nucleotide 20 of the intact L IVS RNA. As regards positions 23, 24, 25 etc., these are positions 23, 24, 25 of the L IVS RNA (as if the first 19 positions were present).

Since dC₅ binds to the L-19 IVS RNA (see above), it is likely that the endoribonucleases will work on mixed polymers of RNA and DNA. For example, L-19 IVS RNA will bind the DNA portion while the RNA enzyme works on the RNA piece of the mixed polymer. Alteration of the binding site to bind the other nucleotides will result in an array of mixed polymer activity in a series of such endoribonucleases.

Abbreviations: IVS, intervening sequence; L-19 IVS RNA (read "L minus 19"), a 395-nucleotide linear RNA missing the first 19 nucleotides of the IVS; CHES, 2-(cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MES, 2-(N-Morpholino)-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; p*, ³²P within an oligonucleotide (for example, C₅p* is CpCpCpCpCp[³²P]-pCp).

Enzyme Preparation. L-19 IVS RNA can be synthesized and purified as described by Zaug and Cech (1986) Science (Wash., D.C.) 231:470-475 (see Fig. 5 for detailed description). In brief, RNA was transcribed from pSPTT1A3 with bacteriophage SP6 RNA polymerase in vitro. (Alternatively RNA can be transcribed from pT7-TT1A3 or from any of the plasmids in the pBG series with bacteriophage T7 RNA polymerase in vitro). Transcripts were further incubated to promote self-splicing and cyclization of the IVS RNA. The RNA was subsequently incubated in MgCl₂ at pH 9.0 (site-specific hydrolysis conditions) to convert circular IVS RNA to L-19 IVS RNA. The L-19 IVS RNA was purified by polyacrylamide gel electrophoresis and Sephadex G-50 chromatography. Enzyme concentration was determined by spectrophotometry assuming a molar extinction coefficient at 260nm of 3.26 x 10⁶ M⁻¹ cm⁻¹.

Active plasmids in use are as follows:

pSPTT1A3, pT7-TT1A3, pBGST7, pBG/-2G:23C, pBG/23C, pBG/-3G:24C, pBG/24C, pBG/-4G:25C, pBG/25C, and pBG/23A, and pT7L-21. The pBG plasmid series is described in Been, M. and Cech, T.R. (1986) Cell 407:207. For example, the pBG/3G:24C and the pBG/24C plasmids produce the L-19 IVS RNA

24C variant which cleaves RNA after the CGCU 4 base sequence. These plasmids are on deposit and available at the Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215. Examples of these including pBG-ST7 (ATCC 40288), pT7-TT1A3 (ATCC 40290) and pBG-3G:24C (ATCC 40289) have been deposited with the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville Maryland 20301 on November 25, 1988. The plasmid pT7L-21 makes the L-21 IVS RNA wherein the first 21 bases are deleted. This plasmid (ATCC 40291) was also placed on deposit at the ATCC on December 2, 1988.

Preparation of Substrates. C_5p^*Cp and A_6p^*Cp were prepared from C_5-OH and A_6-OH , respectively, with T_4 RNA ligase (New England Nuclear), p^*Cp , and ATP. Products were purified by 20% polyacrylamide-7 M urea gel electrophoresis and Sephadex G-25 chromatography. C_5p^* was prepared from C_5p^*Cp by treatment with calf intestinal phosphatase and beta-elimination (Winter & Brownlee, 1978 Nucleic Acid. Res. 5:3129). Unlabeled C_5p was prepared in a similar manner with unlabeled pCp as donor in the ligase reaction. Concentration was determined by spectrophotometry using a molar extinction coefficient at 270nm of $30 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Preparation of E- p^* . Unlabeled L-19 IVS RNA (16 pmol) was incubated with 5.2 pmol of C_5p^* in 50 mM NaOAc, pH 5.0, and 20 mM $MgCl_2$ at 42°C for 10 min. The reaction was stopped by the addition of EDTA to 40 mM. The E- p^* was purified from unreacted C_5p^* by column chromatography on Sephadex G-100-120, which was equilibrated in 0.01 M Tris-HCl, pH 7.5, 0.25 M NaCl, and 0.001 M EDTA. The fractions that contained E- p^* complex were pooled and precipitated with 3 volumes of ethanol. The dried precipitate was then dissolved in H_2O .

Standard Endoribonuclease Reactions

Substrate RNA is pretreated with glyoxal according to the procedure of Carmichael and McMaster (1980) Meth in Enzymol. 65:380-391 and then ethanol precipitated and the precipitate pelleted by centrifugation in an eppendorf centrifuge. The pellet is dried and the RNA re-suspended in water. The glyoxylated substrate RNA (0.2 μM) is incubated with ribozyme (for example L-19 IVS-beta RNA, 0.2 μM) at 50°C in 10 mM $MgCl_2$, 10 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.5 mM GTP, 2.5M Urea for 1 hour.

Stopping Reactions and Analyzing Products

In all cases reactions are stopped by the addition of EDTA to a final concentration of 25 mM. Products can be analyzed by electrophoresis in a 20% polyacrylamide, 7.0 M Urea gel (standard sequencing gel). If ^{32}P -labelled RNA substrates are used, products can be localized by autoradiography.

The following Examples and the standard conditions above serve to illustrate, but not to limit the invention.

Example 1

Sequence-specific cleavage of large RNAs:

The L-19 IVS RNA enzyme was prepared by incubation of pre-rRNA under conditions that promote self-splicing, cyclization, and site-specific hydrolysis, (Zaug, A.J., et al. (1984) Science 224:574; Zaug, A.J., et al. (1986) Science 231:470). The 3'-terminal guanosine (G^{414}) was then removed from the L-19 IVS RNA by periodate oxidation followed by beta-elimination (Winter, G., et al. (1978) Nucleic Acids Res. 5:3129-3139). As expected, the resulting ribozyme (L-19 IVS-beta) has greatly reduced activity as a nucleotidyl-transferase, assayed using $[^{32}P]-p(C)_5$ as a substrate. When GTP was added, however, the ribozyme was able to cleave $p(C)_5$ as well as large RNA molecules. For example, the 504 nt pAK105 transcript (Mount, S.M., et al. (1983) Cell 33:509-518), a fragment of mouse beta-globin pre-mRNA containing the first intron, was cleaved to give major fragments of 148, 360 and 464 nt, as well as some minor fragments (Fig. 2a). As shown below, the 360 and 148 nt fragments can be explained as the 5' and 3' products of cleavage at position 360. The 464 nt fragment is the 3' product of cleavage at position 44, the 5' 44 nt fragment being too small to be observed. The absence of a major amount of a 316 nt RNA, the expected product of cleavage at both position 44 and 360, is indicative of partial digestion with few molecules cleaved more than once.

Cleavage required magnesium ion (optimum at 10-20 mM $MgCl_2$) and was essentially independent of monovalent cation in the range 0-200 mM NaCl. The pH optimum was in the range of 7.5-8.0, and the temperature optimum was approximately 50°C. Although the beta-eliminated L-19 IVS RNA was competent

to catalyze the cleavage reaction, removal of G^{14} from the ribozyme was not required for cleavage activity. The enzyme worked at the same rate whether or not G^{14} had been removed. We explain the activity of the intact L-19 IVS RNA by the postulate that, at saturating concentrations of GTP, the attack by GTP on the substrate competes very effectively with attack by G^{14} .

- 5 We note the IVS RNA and L-19 IVS RNA are protein-free. The L-19 IVS RNA is therefore a protein-free RNA enzyme (or ribozyme). L-19 IVS RNA and the like also function as enzymes in the absence of proteins. This applies to exogenous as well as endogenous protein. This is evidenced by retention of activity when subjected to protease activity, boiling or sodium dodecyl sulfate. Any RNA polymerase protein from the transcription system used to produce IVS RNA is removed by phenol extraction. The ability to
10 make the IVS RNA in a totally defined system in vitro, and remove the RNA polymerase by phenol extraction is further evidence of the protein-free nature of the reaction.

Example II

- 15 Labelled cleavage products:

When [α - 32 P]GTP was included in the reaction of pAK105 RNA as in Example I above, the 148 and 464 nt cleavage products were labeled (Fig. 2b). Direct sequencing of these labeled RNA fragments (e.g., Fig. 2c) showed that cleavage and GTP-addition occur at nucleotides 44 and 360 in the sequence, such that
20 the downstream cleavage products are 5'-GTP labeled. The bonds formed by GTP addition are sensitive to RNase T₁, confirming that the GTP was covalently added through its 3'-O by a normal 3'-5' phosphodiester bond. Reaction of the 841 nt pT7-1 (Xmn 1) RNA (essentially pBR322 sequences) produced 4 major labeled fragments. These were sequenced in the same manner. pT7-1 RNA with an additional 122 nt at its 3' end, produced by transcription of pT7-1 DNA that had been cleaved at the Sca I site, showed the
25 expected increase in molecular weight of the labeled products. In all of the reactions, including those in which substrate RNA was omitted, the L-19 IVS RNA became labeled, perhaps by the guanosine-exchange reaction proposed elsewhere (Zaug, A.J., et al. (1985) Science 229:1060-1064; Price, J.V., et al. (1987) J. Mol. Biol. in press). The sites of self-labeling were heterogeneous.

- 30 Example III

Specificity Assessment:

The sequence near the 5' end of each end-labeled product (See Example II above) was compared to
35 the known sequence of the RNA to identify the nucleotides preceding the site of cleavage. The results are summarized in Table 1. Both the major and the minor cleavage sites are preceded by four pyrimidines, the consensus sequence being CUCU. This is exactly the tetranucleotide sequence expected to be an optimal target for the ribozyme. The importance of nucleotides at positions -5 and -6 relative to the cleavage site is not yet clear, although the absence of G residues may be significant. There is no apparent sequence
40 preference downstream from the cleavage site, with all four nucleotides represented at position +1.

In assessing specificity, it is also necessary to consider which potential sites in the RNA were not cleaved by the ribozyme. For the pAK105 RNA, there was only one CUCU site at which cleavage was not observed. (Cleavage at this site would have produced a labeled 378 nt RNA.) On the other hand, a great many sites that match CUCU in 3 out of 4 positions were not cleaved. These include 17 CUMU sequences
45 (where M \neq C) and 7 CNCU sequences (where N \neq U). In pT7-1 RNA, cleavage was observed at both the CUCU sequences in the RNA, but at only one of the 15 UUUU sequences present. Thus, the ribozyme has a strong preference for cleavage after the tetranucleotide CUCU.

Example IV

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Cleavage within regions of base-paired RNA secondary structure:

M1 RNA, the RNA subunit of *E. coli* RNase P (Reed, R.E., et al. (1982) Cell 30:627-636), was not
55 cleaved by L-19 IVS RNA-beta under standard reaction conditions (Fig. 2b). M1 RNA contains the sequence UCCUCU, which should be an excellent target site. However, this sequence is involved in a stable hairpins stem (Guerrier-Takada, C., et al. (1984) Biochemistry 23:6327-6334; Pace, N.R., et al. (1985) Orig. of Life 16:97-116), which presumably makes it unavailable as a substrate. We have found that denaturation of M1 RNA with glyoxal allowed efficient cleavage of this site by L-19 IVS RNA, in support of the interpretation

that RNA secondary structure can inhibit cleavage. The glyoxal procedure used for denaturation is according to Carmichael, G.G., et al. (1960) *Meth. in Enzymol.* 65:380-391.

Example V

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Active-site mutations alter substrate specificity:

The substrate specificity was next studied in a defined system where we could be certain that secondary structure in the substrate RNA was not affecting the cleavage reaction. Oligoribonucleotide
10 substrates were synthesized by the phage T7 RNA polymerase transcription method developed by Uhlenbeck and co-workers (Lowary, P., et al. (1988) NATO ASI Series, vol. 110, 69-76) (Fig. 3a). One substrate contained a perfect match to the tetranucleotide consensus sequence. Two other substrates had single-base changes giving a 3-out-of-4 match to the consensus.

These substrates were tested with the wild-type L-19 IVS RNA and with two altered ribozymes (Been, M.D. and Cech, T.R., (1988) *Cell* 47:207-216). The two variants have single-base changes in the 5' exo-binding site that alter the sequence specificity of the first step in pre-rRNA self-splicing (Been, M.D., et al. Supra). The 23C variant (G converted to C at position 23 of the L-19 IVS RNA) is expected to recognize CUGU substrates, and the 24C (A converted to C at position 24) variant should recognize CGCU (Fig. 3b). In the course of these studies, we found that the inclusion of 2.5M urea or 15% formamide in the reactions
20 greatly increased the specificity, allowing each ribozyme to differentiate substrates with a single base change in the recognition sequence. Our operating model is that these denaturants destabilized the base-pairing between the substrate and the active site nucleotides of the ribozyme, thereby discriminating against mismatched complexes. The results of treatment of the 3 substrates with each of the 3 ribozymes in the presence of 2.5 M urea are shown in Fig. 3c. Each substrate is cleaved exclusively by the ribozyme that is
25 capable of making a perfectly base-paired enzyme-substrate complex (Fig. 3b). Thus it is contemplated the active site can be manipulated to recognize any base sequence so far that is XYZU, where X, Y and Z can be any of the four bases A,U,C,G and the nucleotides in the four base sequence can be the same or different.

When variant ribozymes were incubated with the 504 nt pAK105 RNA, each ribozyme gave a different pattern of cleavage products. One major cleavage site has been mapped for three variant ribozymes, including a 25C variant (G converted to C at position 25). The sites cleaved by the 23C, 24C and 25C ribozymes are preceded by CCCUGU, UCUGCU, and CUGUCU, respectively; the underlining indicates the base that would form a G-C base pair with the mutated nucleotide in the variant ribozyme. Each of these sequences can form 6 continuous base-pairs with the active site nucleotides of the variant ribozyme. While
30 more cleavage sites must be sequenced before specificity can be properly assessed, these initial results are promising.

Example VI

40 Cleavage is catalytic:

The time course of cleavage of the oligonucleotide substrate GGCCUCU*AAAAA (where the asterisk designates the cleavage site) by the wild-type ribozyme is shown in Fig. 4a. The reaction of 2.5 uM substrate with 0.2 uM ribozyme is 66% complete in 90 minutes. Thus, it is readily apparent that the
45 ribozyme is acting catalytically.

The reaction rate was determined at a series of substrate concentrations. The kinetics are adequately described by the Michaelis-Menten rate law. The dependence of the rate on GTP concentration is shown in the form of a Lineweaver-Burk plot in Fig. 4b. The K_m for GTP is 44 uM. The dependence of the rate on RNA substance concentration at saturating GTP concentration is shown in Fig. 4c. The K_m for this
50 oligoribonucleotide substrate is 0.8 uM, and k_{cat} is 0.13 min⁻¹. Thus under V_{max} conditions the enzyme turns over about 8 times per hour.

Plasmid Construction

55 Been, Michael D. and Cech, Thomas, R. (1986) *Cell* 47:207-216 reports the construction of the pBG plasmid series. In general, the plasmid used for these studies (pBGST7) was derived from the cloning vector pUC18. The methodology for the following manipulations is described in Maniatis, T., et al. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York: Cold Spring Harbor). pUC18 was

partially digested with HaeII and unit length linear molecules were isolated by agarose gel electrophoresis and electroelution. The recovered DNA was treated with T4 DNA polymerase to make blunt ends and then dephosphorylated with calf intestinal phosphatase. pT7-2 (U.S. Biochemical Corp.) was cut with PvuII and HindIII and treated with T4 DNA polymerase, and the fragment containing the phage T7 promoter was isolated by polyacrylamide gel electrophoresis and electroelution. The promoter-containing fragment was ligated into the linearized pUC18 and the plasmid transformed into *E. coli* strain JM83. Individual colonies were picked and miniprep DNA was screened by restriction endonuclease digestion to locate the promoter fragment to the correct HaeII site (position 680 on the map in the New England Biolabs Catalog). The orientation of the T7 promoter was determined by transcribing EcoRI-cut miniprep DNA with T7 RNA polymerase and determining the size of the product. This plasmid (pUT718) was then cut in the multicloning site with KpnI and SphI and treated with T4 DNA polymerase followed by calf intestinal phosphatase. An IVS-containing BamHI DNA fragment was isolated from pJE457 (Price, J.V. and Cech, T.R., (1985) *Science* 228:719-722) and treated with S1 nuclease, phenol extracted, chloroform-extracted, and ethanol precipitated. The S1-treated fragment, containing short deletions at both ends, was ligated into the KpnI/SphI-cut pUT718. *E. coli* strain JM83 was transformed with the recombinant plasmid and plated on LB agar containing ampicillin and X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside). DNA isolated from blue colonies was assayed by agarose gels electrophoresis for IVS-size inserts. Exon sequences were determined by dideoxy-sequencing the miniprep DNA. Ideally, only plasmids containing IVS and exons that will preserve the reading frame of the lacZ gene fragment should give blue colonies and this will depend on the splicing of the message in *E. coli* (Waring, R.B., et al., (1985) *Cell* 40:371-380; Price, J.V. and Cech, T.R., (1985) *Science* 228:719-722). In fact, several plasmids that conferred a light blue color to the colonies had exons for which correct splicing of the IVS should not generate the proper reading frame; these were not investigated further. One that produced a dark blue colony, pBGST7 (Figure 5), also had the expected reading frame and was used for these studies.

Mutagenesis

Oligonucleotide-directed mutagenesis on plasmid DNA was done essentially as described by Inouye, S. and Inouye M. (1988) In *DNA and RNA Synthesis*, S. Narang, ed; (New York: Academic Press), in press. pBGST7 DNA was cleaved in the gene coding for ampicillin resistance with XmnI. In a separate reaction, pBGST7 DNA was cut with EcoRI and HindIII, sites that flank the IVS and exon sequences. The vector portion of the plasmid was separated from the IVS-containing fragment by agarose gel electrophoresis and recovered by electroelution. XmnI-cut DNA was mixed with the vector fragment in the presence of the oligonucleotide containing the mismatched base, heated to 95 °C for 5 min, placed at 37 °C for 30 min, then at 4 °C for 30 min and then put on ice. The products were treated with dNTPs and Klenow fragment of DNA pol I at room temperature for 2 hr. *E. coli* strain JM83 was transformed with the DNA and ampicillin-resistant colonies were picked on the basis of color (white or light blue indicating loss of splicing activity) and the miniprep DNA was sequenced.

The double mutants were made using the plasmid DNA of single mutants and screening for restored beta-galactosidase activity. pBG/-2G:23C was made from pBG/-2G and a second oligonucleotide directed at position 23. pBG/-3G:24C was made starting with pBG/24C and using the oligonucleotide directed at position -3. pBG/-4G:25C was made by generating a circular heteroduplex from pBG/-4G:25C that had been linearized at different positions. Transformation of these heteroduplexes generated blue and light blue colonies, both at a low frequency. The blue colonies were wild-type sequence the light blues double mutant.

pSPTT1A3:

The Thal fragment of *Tetrahymena* ribosomal RNA gene contains the IVS and small portions of the flanking exons. Hind III linkers were ligated onto the ends of the Thal fragment and it was inserted into the Hind III site in the polylinker of pSP62 (New England Nuclear), which contains the SP6 promoter. The recombinant plasmid was cloned in *E. coli* by standard methods.

pT7TT1A3:

Construction of pT7TT1A3 was identical to pSPTT1A3 except that the Thal fragment was cloned into the Hind III site of pT7-2 (U.S. Biochemical Corp.) which contains the T7 promoter.

pT7 L-21 plasmid:

pBGST7 (described in Been & Cech, (1986) Cell 47:207) was cleaved with restriction endonucleases SphI and Hind III and the small fragment containing the IVS minus its first 44 nucleotides was purified. pUC18 was cleaved with Sph I and Hind III, mixed with the Sph-Hind III fragment from pBGST7 and treated with DNA ligase. E. coli cells were transformed, colonies were picked, plasmid DNA was isolated from individual colonies and sequenced. A plasmid containing the Sph-Hind III fragment of the IVS properly inserted into pUC18 was selected for the next step. This plasmid was cleaved with SphI and EcoRI restriction endonucleases and a synthetic oligonucleotide was inserted into the plasmid using DNA ligase. The synthetic oligonucleotide contained one-half of the EcoRI restriction site, the T7 promoter, and nucleotides 22-44 of the *Tetrahymena* IVS, ending at the SphI site. E coli cells were transformed, colonies were picked, plasmid DNA was isolated from individual colonies and sequenced.

The final plasmid pT7L-21 was found by sequence analysis to contain the T7 promoter properly juxtaposed to nucleotide 22 of the IVS. (See Figure 5)

Therefore the method can be used to create defined pieces of RNA. For example, these defined pieces can be 5'-OH or 3'-OH end pieces, or center pieces containing the active site for study and production of variant RNA forms.

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Table 1. Sites of cleavage of large RNA substrates by the wild-type L - 19 IVS_β RNA

Substrate	Site	Size (nt) [*]	Sequence ⁺	
			-6	-1
pAK-105 (β-globin pre-mRNA)	1	148	UCCUCU	↓GCCUC
	2	464	AACUCU	AAGAG
pT7-1 (pBR322)	1	145	UCCCUU	UUUUG
	2	556	CACUCU	CAGUA
	3	603	UAGUUU	CUCCU
	4	805	UCCUCU	AGAGU
Consensus				
observed			U ^C ACUCU	↓N
expected ⁺⁺			C ^C UCUCU	↓N

* Size of the GTP-labeled Fragment.

⁺ Sequences are listed in 5' 3' direction. Arrows indicate sites of cleavage and guanosine addition.

⁺⁺ The expected consensus sequence is based on the known sequence at the end of the 5' exon (CUCUCU) modified by the possibility that either a C or a U at position -5 might be able to pair with the G in the active site. At position -1, there is some basis for thinking that a C might not be as good as a U (Davies, R.W., et al. (1982) Nature 300:710-724; Michel, F., et al. (1983) EMBO J. 2:33-38; Inoue, T., et al. (1986) J. Mol. Biol. 189:143-165.

Claims

1. An enzymatic ribonucleic acid molecule which is capable of cleaving by transesterification a separate RNA molecule at a predetermined phosphate ester bond in a single-stranded target nucleotide sequence within the separate RNA molecule, which enzymatic molecule comprises:
 - (i) a substrate binding portion capable of binding with the target nucleotide sequence; and
 - (ii) an enzymatic portion having endonuclease activity independent of any protein *in vitro*, wherein said enzymatic molecule can only cleave the single-stranded target sequence without forming a covalent bond between said enzymatic molecule and any portion of said separate RNA molecule.

2. An enzymatic molecule according to claim 1 in purified form.
3. An enzymatic molecule according to claim 1 or 2 wherein the substrate binding portion comprises four or more bases.
- 5 4. An enzymatic molecule according to any of claims 1 to 3 which has a pH optimum for said endonuclease activity between 7.5 and 8.0.
- 10 5. An enzymatic molecule according to any of claims 1 to 4 wherein said enzymatic nucleic acid molecule further comprises a second endonuclease activity, independent of any protein, for nucleic acid comprising deoxynucleotides.
6. An enzymatic molecule according to any of claims 1 to 5 which has been chemically synthesised.
- 15 7. A method for specifically cleaving a separate RNA molecule at a target nucleotide sequence comprising single-stranded RNA, comprising contacting an enzymatic ribonucleic acid molecule as defined in any one claims 1 to 6 with a separate RNA molecule which contains a single-stranded target nucleotide sequence under conditions to cause specific cleavage of the target sequence by endonuclease activity of said enzymatic molecule.
- 20 8. A method according to claim 7 wherein the cleavage is carried out in the presence of guanosine or a phosphate ester thereof.
9. A method according to claim 7 or 8 wherein the cleavage is carried out in the presence of a divalent cation.
- 25 10. A method according to claim 9 wherein the divalent cation is Mg^{2+} .
11. A method for producing an enzymatic ribonucleic acid molecule having an endonuclease activity independent of any protein, said activity being specific for a nucleotide sequence defining a cleavage site comprising a single-stranded RNA target site in a separate first RNA molecule, comprising the steps of:
 - (i) identifying a second RNA molecule capable of intramolecular cleavage by transesterification resulting from nucleophilic attack at a phosphorous ester bond,
 - 35 (ii) determining the cleavage site and nucleophilic attacking group of said second RNA molecule, said site comprising at least four bases, and
 - (iii) synthesising an enzymatic ribonucleic acid molecule corresponding to said second RNA molecule but lacking said cleavage site and said nucleophilic attacking group, such that said enzymatic molecule can only cleave by transesterification, a single-stranded target sequence without forming a covalent bond between itself and any portion of the target sequence, wherein said
 - 40 synthesising optionally includes altering one or more residues of said second RNA molecule such that the target specificity of the enzymatic molecule is altered.
12. The method of claim 11 wherein said determining step comprises determining the nucleotide immediately adjacent in the 3' or 5' direction to said cleavage site of said second RNA molecule.
13. The method of claim 11 or 12 further comprising determining the sequence in said second RNA molecule that base-pairs with the nucleotide sequence comprising said cleavage site and retaining the sequence in said second RNA molecule while removing the sequence that base-pairs with it.

Patentansprüche

1. Enzymatisches Ribonucleinsäuremolekül welches in der Lage ist, durch Umesterung, ein separates RNA-Molekül an einer vorbestimmten Phosphatesterbindung in einer einzelsträngigen Ziel-Nucleotidsequenz innerhalb des separaten RNA-Moleküls zu spalten, wobei das enzymatische Molekül folgendes umfaßt:
 - (i) einen Substratbindungsteil, der zur Bindung mit der Ziel-Nucleotidsequenz in der Lage ist; und

(ii) einen enzymatischen Teil mit Endonucleaseaktivität, unabhängig von irgendeinem Protein *in vitro*, wobei das enzymatische Molekül nur die einzelsträngige Zielsequenz spalten kann, ohne Bildung einer kovalenten Bindung zwischen dem enzymatischen Molekül und irgendeinem Teil des separaten RNA-Moleküls.

- 5 2. Enzymatisches Molekül nach Anspruch 1 in gereinigter Form.
3. Enzymatisches Molekül nach Anspruch 1 oder 2, wobei der Substratbindungsteil vier oder mehr Basen beinhaltet.
- 10 4. Enzymatisches Molekül nach mindestens einem der Ansprüche 1 bis 3, welches ein pH-Optimum bezüglich der Endonucleaseaktivität zwischen 7,5 und 8,0 besitzt.
- 15 5. Enzymatisches Molekül nach mindestens einem der Ansprüche 1 bis 4, wobei das enzymatische Nucleinsäuremolekül ferner eine zweite von irgendeinem Protein unabhängige Endonucleaseaktivität für Desoxynucleotide beinhaltende Nucleinsäure besitzt.
- 20 6. Enzymatisches Molekül nach mindestens einem der Ansprüche 1 bis 5, welches chemisch synthetisiert worden ist.
- 25 7. Verfahren zur spezifischen Spaltung eines separaten RNA-Moleküls an einer einzelsträngigen RNA beinhaltenden Ziel-Nucleotidsequenz, umfassend das Kontaktieren eines enzymatischen Ribonucleinsäuremoleküls, wie es nach mindestens einem der Ansprüche 1 bis 6 definiert ist, mit einem separaten RNA-Molekül, welches eine einzelsträngige Ziel-Nucleotidsequenz enthält unter Bedingungen, die zu einer spezifischen Spaltung der Zielsequenz durch Endonucleaseaktivität des enzymatischen Moleküls führen.
8. Verfahren nach Anspruch 7, wobei die Spaltung in Gegenwart von Guanosin oder eines Phosphatesters davon durchgeführt wird.
- 30 9. Verfahren nach Anspruch 7 oder 8, wobei die Spaltung in Gegenwart eines zweiwertigen Kations durchgeführt wird.
- 35 10. Verfahren nach Anspruch 9, wobei das zweiwertige Kation Mg^{2+} ist.
- 40 11. Verfahren zur Herstellung eines enzymatischen Ribonucleinsäuremoleküls mit einer von irgendeinem Protein unabhängigen Endonucleaseaktivität, wobei die Aktivität für eine Nucleotidsequenz spezifisch ist, die eine Spaltstelle definiert, welche eine einzelsträngige RNA-Zielstelle in einem separaten ersten RNA-Molekül beinhaltet, umfassend die Schritte:
 - (I) Nachweisen eines zweiten RNA-Moleküls, das zur intramolekularen Spaltung durch Umesterung, die sich aus einem nucleophilen Angriff an einer Phosphoresterbindung ergibt, in der Lage ist,
 - (II) Bestimmen der Spaltstelle und der nucleophilen Angriffsgruppe des zweiten RNA-Moleküls, wobei jene Stelle mindestens vier Basen beinhaltet, und
 - (III) Synthetisieren eines enzymatischen Ribonucleinsäuremoleküls, das dem zweiten RNA-Molekül entspricht, jedoch nicht die Spaltstelle und die nucleophile Angriffsgruppe besitzt, so daß das enzymatische Molekül nur durch Umesterung eine einzelsträngige Zielsequenz spalten kann, ohne Bildung einer kovalenten Bindung zwischen sich und irgendeinem Teil der Zielsequenz, wobei das Synthetisieren gegebenenfalls das Abändern eines oder mehrerer Reste des zweiten RNA-Moleküls einschließt, so daß die Zielspezifität des enzymatischen Moleküls verändert ist.
- 45 12. Verfahren nach Anspruch 11, wobei der Bestimmungsschritt das Bestimmen des Nucleotids in direkter Nachbarschaft in der 3'- oder 5'-Richtung der Spaltstelle des zweiten RNA-Moleküls umfaßt.
- 50 13. Verfahren nach Anspruch 11 oder 12, ferner umfassend das Bestimmen der Sequenz in dem zweiten RNA-Molekül, die mit der die Spaltstelle beinhaltenden Nucleotidsequenz eine Basenpaarung eingeht, und Behalten der Sequenz in dem zweiten RNA-Molekül, während die Sequenz, die damit Basenpaarung eingeht, entfernt wird.
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Revendications

1. Molécule d'acide ribonucléique enzymatique qui est capable de cliver par transestérification une
 5 molécule d'ARN séparée, au niveau d'une liaison ester phosphate prédéterminée, dans une séquence
 nucléotidique cible simple brin comprise dans la molécule d'ARN séparée, molécule enzymatique qui
 comprend :
 (i) une partie pour liaison à un substrat, capable de se lier avec la séquence nucléotidique cible; et
 (ii) une partie enzymatique ayant une activité de type endonucléase indépendante de toute protéine
in vitro;
 10 où ladite molécule enzymatique peut uniquement cliver la séquence cible simple brin sans former
 une liaison covalente entre ladite molécule enzymatique et une partie quelconque de ladite molécule
 d'ARN séparée.
2. Molécule enzymatique selon la revendication 1 sous forme purifiée.
- 15 3. Molécule enzymatique selon la revendication 1, ou 2 dans laquelle la partie pour liaison à un substrat
 comprend quatre bases ou plus.
4. Molécule enzymatique selon l'une quelconque des revendications 1 à 3 qui a un optimum de pH pour
 20 ladite activité de types endonucléase entre 7,5 et 8,0.
5. Molécule enzymatique selon l'une quelconque des revendications 1 à 4 dans laquelle ladite molécule
 d'acide nucléique enzymatique comprend en outre une seconde activité de type endonucléase,
 indépendante de toute protéine, vis-à-vis d'un acide nucléique comprenant des désoxynucléotides.
- 25 6. Molécule enzymatique selon l'une quelconque des revendications 1 à 5 qui a été produite par synthèse
 chimique.
7. Méthode pour cliver spécifiquement une molécule d'ARN séparée au niveau d'une séquence nucléotidi-
 30 que cible, comprenant un ARN simple brin, comprenant une étape consistant à mettre en contact une
 molécule d'acide ribonucléique enzymatique, telle que définie dans l'une quelconque des revendica-
 tions 1 à 6, avec une molécule d'ARN séparée qui contient une séquence nucléotidique cible simple
 brin dans des conditions entraînant un clivage spécifique de la séquence cible sous l'effet de l'activité
 de type endonucléase de ladite molécule enzymatique.
- 35 8. Méthode selon la revendication 7 dans laquelle le clivage est réalisé en présence d'une guanosine ou
 d'un ester phosphate de celle-ci.
9. Méthode selon la revendication 7 ou 8 dans laquelle le clivage est réalisé en présence d'un cation
 40 divalent.
10. Méthode selon la revendication 9 dans laquelle le cation divalent est Mg^{2+} .
11. Méthode de production d'une molécule d'acide ribonucléique enzymatique ayant une activité de type
 45 endonucléase indépendante de toute protéine, ladite activité étant spécifique d'une séquence nucléoti-
 dique qui définit un site de clivage comprenant un site cible d'ARN simple brin dans une première
 molécule d'ARN séparée, comprenant les étapes consistant à :
 (i) identifier une seconde molécule d'ARN capable d'effectuer un clivage intramoléculaire par
 transestérification résultant d'une attaque nucléophile au niveau d'une liaison ester phosphoreux,
 50 (ii) déterminer le site de clivage et un groupe d'attaque nucléophile de ladite seconde molécule
 d'ARN, ledit site comprenant au moins quatre bases, et
 (iii) synthétiser une molécule d'acide ribonucléique enzymatique correspondant à ladite seconde
 molécule d'ARN mais dépourvue dudit site de clivage et dudit groupe d'attaque nucléophile, de telle
 façon que ladite molécule enzymatique puisse cliver uniquement par transestérification, une séquen-
 55 ce ciblée simple brin sans former une liaison covalente entre elle-même et une partie quelconque de
 la séquence cible, où ladite synthèse comprend éventuellement une altération d'un ou plusieurs
 résidus de ladite seconde molécule d'ARN de telle façon que la spécificité de cible de la molécule
 enzymatique soit altérée.

12. Méthode selon la revendication 11 dans laquelle ladite étape de détermination comprend la détermination du nucléotide immédiatement adjacent dans la direction 3' ou 5' audit site de clivage de ladite seconde molécule d'ARN.

- 5 13. Méthode selon la revendication 11 ou 12 comprenant en outre les étapes consistant à déterminer la séquence dans ladite seconde molécule d'ARN dont les bases s'apparient avec la séquence nucléotidique comprenant ledit site de clivage et à conserver la séquence dans ladite seconde molécule d'ARN tout en éliminant la séquence dont les bases s'apparient avec elle.

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Fig. 1A.

Self-splicing

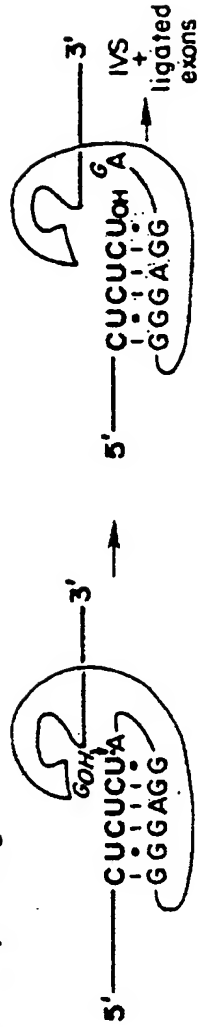
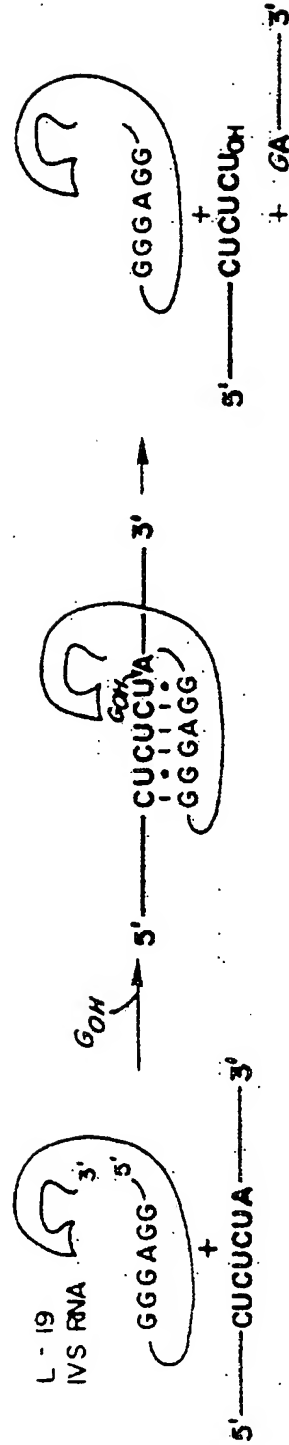
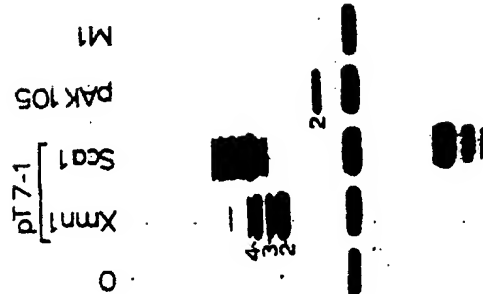
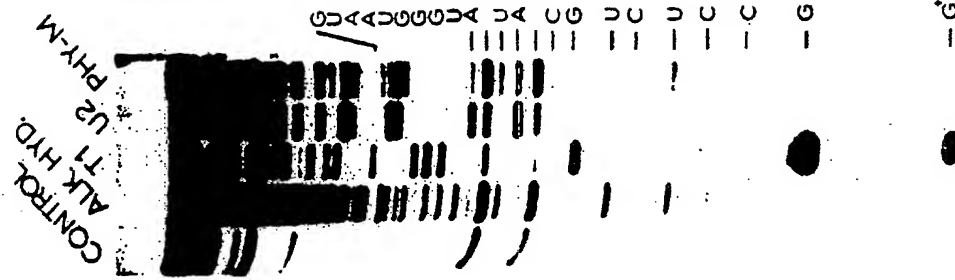


Fig. 1B.

RNA "restriction endonuclease"





— pT7-1(Sca 1)
— pT7-1(Xmn 1)
— pAK 105
— L-19 IVS
— M1 RNA

0 0.1 0.5 M

508—
464—
360—

Fig. 2A.

Fig. 2B.

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Fig. 2C.

Fig. 3A.

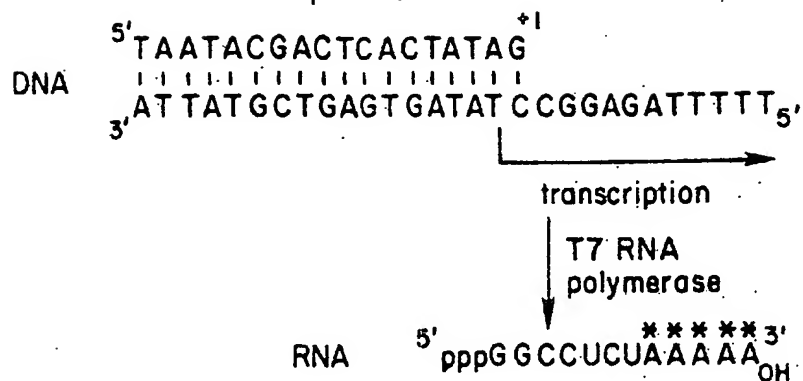


Fig. 3B.

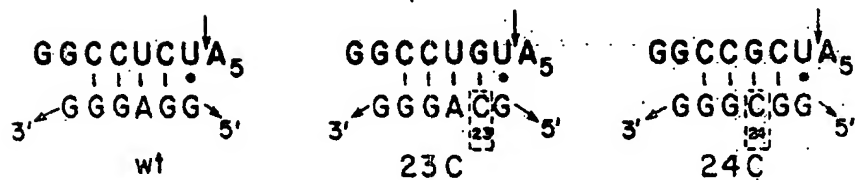


Fig. 3C.

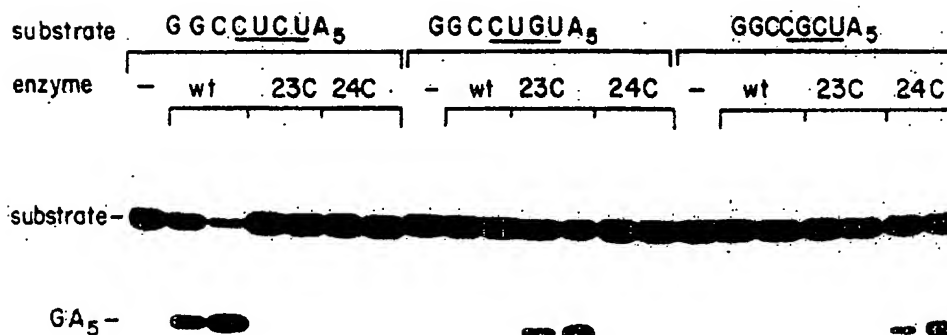


Fig. 4A.

- 0 1 2 5 10 30 60 90 min

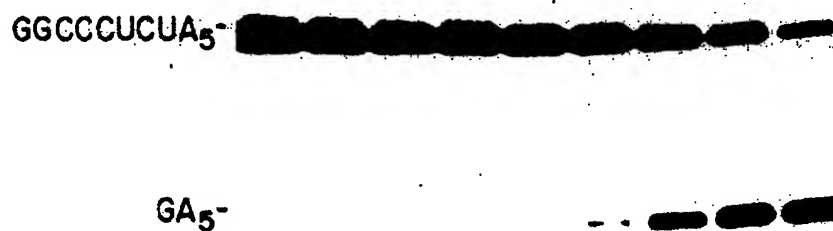


Fig. 4B.

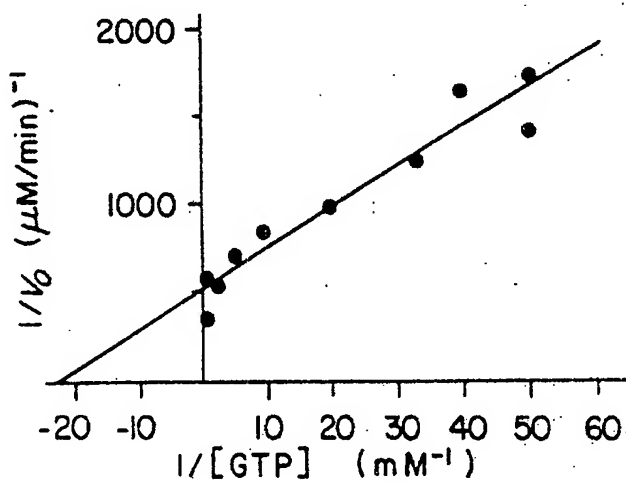


Fig. 4C.

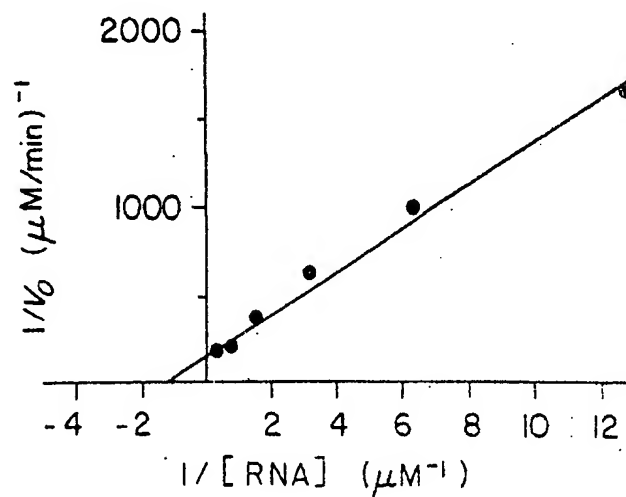
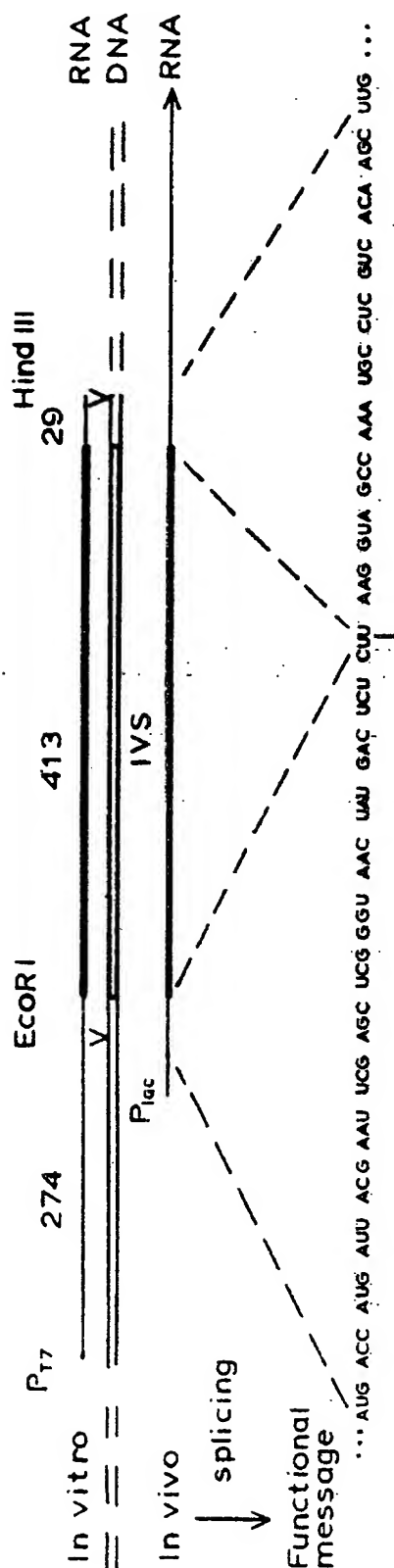


Fig. 16.



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